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MINIREVIEW

Polyhydroxyalkanoate Granules Are Complex Subcellular Organelles (Carbonosomes)[▽]

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Polyhydroxyalkanoates (PHAs) such as poly(3-hydroxybutyrate) (PHB) or poly(3-hydroxyoctanoate), are universal prokaryotic storage compounds of carbon and energy. PHAs are accumulated intracellularly in form of inclusion bodies (PHA granules) during times of oversupply with carbon sources (for reviews, see references 2, 54, 64, 76, 86, and 100). PHAs can consist of short-chain-length hydroxyalkanoic acids (PHA_{SCL}) or medium-chain-length monomers (PHA_{MCL}), depending on strain and culture conditions. Given a number of more than 150 identified hydroxyalkanoates as potential constituents (101) the theoretical number of different PHA copolymers is incredibly high. A few PHAs, such as PHB and copolymers of 3-hydroxybutyrate (3HB), 3-hydroxyvalerate, and/or 4-hydroxybutyrate, are produced by industry (Biocycle, Biomer, Biopol, Enmat, Mirel, and Nodax).

The number of publications on PHA metabolism has considerably increased in the last two decades, and many aspects of the biosynthesis, molecular architecture, intracellular mobilization, extracellular degradation, and commercial applications of PHAs have been addressed. One exiting outcome of these studies was the finding that many polypeptides are specifically present on the surface of PHA granules, much more than would be essential for PHA synthesis. These proteins constitute a particular surface layer on PHA granules that is essential for PHA metabolism. In conclusion, PHA granules are complexly organized subcellular structures and appear to be more than simple polymer inclusions. The current knowledge of the biochemical functions of PHA-associated proteins will be reviewed here. This will be done using the example of *Ralstonia eutropha* H16, which is the model organism of PHA research and has become an important prokaryotic strain for several biotechnological applications (82). It should be noted that several other taxonomic names of *R. eutropha* are found in literature. These include *Hydrogenomonas eutropha*, *Alcaligenes eutrophus*, *Wautersia eutropha*, and *Cupriavidus necator*. We used here the most commonly accepted name, *R. eutropha*, which has been also used for description of the recently sequenced genome (71). When appropriate, the properties of PHA-bound proteins of other organisms are discussed afterward.

PHA SYNTHASES

PHA synthase is the key enzyme of PHA synthesis and catalyzes the polymerization of hydroxyacyl-coenzyme A (CoA) to PHA and free CoA. The first PHA synthase gene (*phaC*) was cloned 20 years ago. Remarkably, this was done independently in three labs with the same bacterial strain, *R. eutrophus* H16 (66, 95, 97). Meanwhile, several PHA synthases from different sources have been biochemically investigated, and many more PHA synthase genes have been cloned or have been determined by genome sequencing. PHA synthases currently are divided into four classes depending on their subunit composition and substrate specificity. The *R. eutropha* and *Pseudomonas putida* (previously *Pseudomonas oleovorans*) PHA synthases represent class I (producing PHA_{SCL}) and class II (producing PHA_{MCL}) synthases, respectively, both of which consist of only the type of subunit with an average subunit size of 60 to 70 kDa. Class III (e.g., *Allochromatium vinosum*, previously *Chromatium vinosum*) and class IV PHA synthases (e.g., *Bacillus megaterium*) consist of two subunits (PhaC/PhaE and PhaC/PhaR, respectively). Thus far, only *Bacillus* sp. have class IV PHA synthases. PHB synthase subunit PhaR of *Bacillus* sp. should not be mixed up with transcriptional regulator PhaR in *R. eutropha* (see below and Table 1). All PHA synthases share a conserved cysteine (Cys_{319} in *R. eutropha* PHA synthase) as a catalytic active site to which the growing PHA chain is covalently attached. The active-site cysteine, along with other conserved amino acids (histidine and aspartate), constitutes a catalytic triad similar to esterases. Comparison of PHA synthase amino acids sequences with sequences of esterases with known structures strongly suggests that PHA synthases have an α/β-hydrolase fold. Details of the molecular events happening during the polymerizing reaction were investigated in several labs and will not be addressed here. The reader should consult the many excellent reviews that were published on this subject in the past (see, for example, references 79–81, 99, 104, 105, and 115).

PHA synthases have been successfully expressed in the cytoplasm of recombinant organisms; however, the specific activity of soluble PHA synthase was significantly lower than that of the PHA granule-bound PHA synthase in in vitro assays using isolated PHA granules. Purified PHA synthase and suitable CoA-activated monomers in an aqueous buffer are sufficient for in vitro synthesis of PHA (17, 39, 41, 78, 91). If the metabolism of a given species is able to provide suitable precursor substrates (acyl-CoAs), recombinantly expressed PHA

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TABLE 1. PHA granule-located proteins

Species and/or strain	PHA	Gene(s)	Protein	Method[a]	Source or reference
Ralstonia eutropha H16	PHB	*phaC* (*phbC*)	PHB synthase	ACT, EM-IG	18, 66, 95, 97
	PHA_{SCL}		PhaC (PhbC)	In the absence of PHB, PhaC is in the soluble fraction	28
		phaP	Phasins: PhaP	WB	74, 118
		phaP1, *phaP3*, *phaP4*	PhaP1, PhaP3, PhaP4, Bkt	2D-PAGE, FM	65, 76
		phaR	PhaR	WB, EM-IG	73
		phaZa1	iPHB depolymerase, PhaZa1	WB	88
		phaZa1-egfp	PhaZa1-EGFP	FM	113
		phaZb (*phaZ2*, *phaY*)	Oligomer hydrolase: PhaZb	WB (partially soluble)	45
		phaZc (*phaY*)	Oligomer hydrolase: PhaZb	WB (partially soluble)	46
		phaZd (*phaZd1*)	PhaZd	WB	1
Ralstonia eutropha PHB-4		*phaC-egfp*	PHB synthase fused to EGFP	FM fusion (PHB granules, located in the cell pole or the periphery; fusion also located in the CM 5 to 10 min after transfer to fresh medium)	D. Jendrossek, unpublished
Ralstonia eutropha/rec. *Escherichia coli*		*phaC-egfp*	PhaC-EGFP	FM (PHB granules, located in the cell pole or the periphery)	67, 68
		buk-ptb-phaEC	HspA BPEC pathway	SDS-PAGE	107
Aeromonas hydrophila	P(3HB-co-3HHx)	*phaP*	PhaP	SDS-PAGE	111
Allochromatium (*Chromatium*) *vinosum*	PHB	*phaCE*	PhaCE	WB, IG-EM	51
			Lysozyme	SDS-PAGE, MALDI	52
Bacillus megaterium	PHB		40- and 22-kDa protein	SDS-PAGE	98
		phaC-gfp	PhaC-Gfp	FM	61
		phaP-gfp	PhaP-Gfp	FM	60
		phaR	PhaR-Gfp	FM	
		phaQ	PhaQ	In vitro binding to aPHB	50
Halophilic *Archaea*	PHB_{SCL}	*phaCE*	PhaCE	WB	22, 53
Magnetospirillum gryphiswaldense	PHB	*mms16-egfp*	Mms16-EGFP	FM	96
Methylobacterium extorquens, *Methylobacterium rhodesianum*	PHB		GA11 protein	SDS-PAGE, WB	9
			GA20 protein	IG-EM	10
Paracoccus denitrificans/ rec. *Escherichia coli*	PHB/PHV	*phaP*, *phaC*, *phaZ*, *phaR*	PhaP, PhaC, PhaZ, PhaR	SDS-PAGE, WB	55, 57
			PhaR-DNA-PHB	Quartz crystal microbalance	56, 121
Pseudomonas putida (*Pseudomonas oleovorans*)	PHA_{MCL}	*phaC*	59-, 55-, 43-, 32-, and 18-kDa polypeptides	SDS-PAGE	12, 14, 102
		phaF, *phaI*	PhaF, PhaI	SDS-PAGE and Edman degradation	77
			PhaF fusions	SDS-PAGE	63, 90
		phaD	PhaD	Location not known; no phasin sensu strictu	42
		phaZ	PhaZ	5	
		acs1-gfp	Acs1-Gfp	FM	87
Rhodococcus ruber/rec. *Escherichia coli*	PHB	*phaP*	(GA14 protein)	SDS-PAGE, IG-EM	69
			PhaP		70
Rhodospirillum rubrum	PHB	*apdA-egfp*	ApdA-EGFP	FM	25
Multiple species and strains	PHA		PhaP homologs and orthologs	SDS-PAGE, WB	119
Allochromatium vinosum	PHA_{SCL}		PHA granules, bound macromolecules	EM studies	58
Pseudomonas putida	PHA_{MCL}				
Rhodococcus ruber	PHA_{SCL}				
Caryophanon latum	PHA_{SCL}				37

[a] Abbreviations: ACT, activity; EM-IG, electron microscopy immunogold technique; WB, Western blotting; FM, fluorescence microscopy; CM, cytoplasmic membrane; MALDI, matrix-assisted laser desorption ionization. rec., recombinant.

synthase catalyzes the formation of PHA in vivo in different host such as plants, animals, or yeasts (49, 72, 120).

PHA synthases are bound to the surfaces of PHA granules in PHA accumulating cells. This has been unequivocally shown by (i) association of PHB synthase activity to isolated native PHB granules (21), (ii) immunogold labeling in combination with electron microscopy (18, 52, 59), (iii) in vivo analysis using PhaC-green fluorescent protein (GFP) fusion analysis (61, 67), and (iv) identification of PhaC by analysis of PHA granule-associated proteins (Table 1). In the absence of accumulated PHA, however, PHB synthase is apparently present in the soluble fraction of *R. eutropha* cell extracts (28). However, the authors of that study speculated that PHB synthase in PHB-free cells could be also associated with the inner side of the cytoplasmic membrane and that PHB synthase became soluble during cell extract preparation. Evidence for a possible association of PHB synthase with the cytoplasmic membrane was obtained by investigation of *R. eutropha* PHB-4 cells (PHB synthase mutant) harboring a plasmid with a *phaC-egfp* fusion (see the section below on PHA granule biogenesis).

PHASINS (PhaPs) AND PhaR

Early investigations on the composition of PHB granules showed that isolated PHB granules consisted of 97.5% of PHB and minor amounts of proteins and phospholipids (20). That study was the basis for the assumption that PHB granules are covered by a layer of phospholipids into which proteins such as PHB synthase and PHB depolymerase are embedded. It was not known whether phospholipids constitute a monolayer or a bilayer on the granule surface. Transmission electron microscopic investigations of *Rhodospirillum rubrum* cells showed

that the thickness of the surface layer of PHB granules was ~4 nm. Since a typical phospholipids bilayer is ~8 nm thick, PHB granules might be covered by a phospholipids monolayer but surely do not have a bilayer (3). Critical analyses of the literature data were in agreement with the assumption of a monolayer of phospholipids and/or proteins on PHA granules (58) and made other models of more complex surface structures unlikely (103). It was not until 1994 that the first PHB granule-located protein aside from PHB synthase or depolymerase was unequivocally identified (70, 98, 118). These granule-associated proteins were named phasins (PhaPs) in analogy to oleosins, which are proteins on the surface of oil globules in some plant cells (98). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of isolated so called native PHB granules (nPHB) previously had indicated the presence of small PHB granule-bound proteins (51). For overview of other PHB-accumulating bacteria, see Table 1. The amount of phasin protein found in PHB-accumulating *R. eutropha* cells was very high, suggesting that the major part of the PHB granule surface is covered by PhaP. Mutants in *phaP* had significantly decreased levels of accumulated PHB, and the PHB granule morphology was changed: one very large PHB granule was synthesized in *phaP* mutants, while overexpression of PhaP led to synthesis of an increased number of very small PHB granules. These results were the basis for the conclusion that PhaP determines the surface-to-volume ratio of PHB granules and prevents the coalescence of PHB granules in vivo by formation of a proteinaceous boundary layer between the hydrophobic polymer and the hydrophilic cytoplasm. Investigation of other PHA-accumulating bacteria revealed that all of them have small proteins (phasins) attached to the surface layer of PHA granules (119). Expression of PhaP in triacylglycerol-accumulating bacteria showed that PhaP is able to bind to oil droplets in vivo (27, 116). At present, it is not known whether phospholipids detected in isolated nPHB granules are also in vivo components of the granule surface layer or whether they represent an experimental artifact during cell extract preparation.

Regulation of PhaP expression and its influence on PHB synthesis has been studied intensively (55–57, 73, 122, 124). Synthesis of PhaP in *R. eutropha* is strictly dependent on PHB accumulation (125). PhaP expression is regulated by repressor PhaR, and mutants in *phaR* produce PhaP constitutively. Interestingly, PhaR in vivo is bound to PHB granules but is also able to bind to DNA upstream regions of *phaP* and *phaR* (73). It is assumed that PhaR is bound by growing PHB granules during PHB accumulation, resulting in a low concentration of soluble PhaR and allowing the expression of more PhaP and PhaR. Once all binding sites for PhaR at the PHB granules are occupied by PhaR and/or PhaP, excess soluble PhaR binds to DNA upstream regions of *phaP* and *phaR*. As a consequence, the expression of PhaP and PhaR is repressed. The regulation of phasin expression and PHB accumulation presumably is similar in *Paracoccus denitrificans* (55–57). Recently, direct evidence for interaction of *P. denitrificans* PhaR with PHB and DNA was obtained by quartz crystal microbalance (121).

Three additional phasin genes (*phaP2*, *phaP3*, and *phaP4*) have been identified aside from *phaP1* in genome sequence of *R. eutropha* (71, 74, 75) (Table 1). Expression of at least one of them, PhaP3, is increased in *phaP1* mutants and might partially compensate a PhaP1 defect (74), but none of the three additional phasins is essential for PHB accumulation. At present, it is not known which particular functions PhaP2, PhaP3, and PhaP4 might have. A possible involvement of PhaP2 and/or PhaP4 in PHA mobilization has been proposed (75). Multiple phasin proteins have been found in other PHA-accumulating bacteria as well (57, 69, 119).

Investigation of PHB granules by atomic force microscopy revealed that the surface of isolated PHB granules from *R. eutropha* is covered by a network or a skeletonlike structure (6). Recent evidence from two labs suggested that PhaP1 is necessary for formation of this structure (48) and possibly is a component of it (7). Interestingly, magnetosomes, which are subcellular structures in magnetotactic bacteria, are organized via a proteinaceous skeletonlike structure (47, 92) to which the magnetosomes are attached. It might be that correct formation of PHB granules is dependent on bacterial cytoskeleton compounds or on other, not-yet-identified structural elements (for a review, see reference 19).

iPHA DEPOLYMERASES

The physiological function of accumulated PHAs in bacteria is to provide nutrients for energy and carbon metabolism during phases of starvation in an osmotically inert form (93, 94). The great importance of accumulated PHAs for survival in the absence of suitable energy/carbon sources was demonstrated many years ago (e.g., for *R. eutropha*) (30). PHB-rich cells of this species are even able to perform one to two cell divisions in the absence of an exogenous carbon source if other nutrients are provided (23). Recently, it was demonstrated that cells of *Legionella pneumophila* with accumulated PHB could survive up to 600 days in tap water (33). Enzymes that catalyze the depolymerization of previously accumulated PHAs are called intracellular PHA (iPHA) depolymerases (PhaZs) (36, 89). iPHA depolymerases have been described first for PHA_{MCL}-accumulating *Pseudomonas putida* (*P. oleovorans*) (5, 11–13) (32). Interestingly, the gene coding for the $iPHA_{MCL}$ depolymerase (*phaZ*) is located between two copies of PHA_{MCL} synthase genes (*phaC1* and *phaC2*) in all investigated PHA_{MCL}-accumulating bacteria. Recently, iPHA depolymerase activity could be demonstrated in vitro by use of radiolabeled substrates ($nPHA_{MCL}$) or by pH stat ($nPHA_{SCL}$), respectively (5, 16). iPHB depolymerases of PHA_{SCL}-accumulating bacteria were not described before 2000, but in the first 5 years of this century no fewer than seven putative iPHB depolymerases and two 3HB oligomer hydrolases have been postulated for *R. eutropha* (1, 23, 44–46, 71, 88, 123). Depending on similarities of the primary amino acid sequences to each other these hydrolases were named PhaZa1 to PhaZa5 and PhaZd1 and PhaZd2 for the iPHB depolymerases and PhaZb and PhaZc for the oligomer hydrolases, respectively. Alternative names for PhaZb and PhaZc are PhaY1 and PhaY2 (71). Unfortunately, assay of iPHA depolymerase is difficult, because of the nature of the polymeric substrate: in vivo, PHAs are amorphous and are covered by a surface layer consisting of proteins (mainly phasins) and perhaps phospholipids (see above). In many research labs, the activity of iPHB depolymerases is assayed by using artificial detergent-coated (cholate, oleate) PHB granules (aPHB) as a substrate that were prepared from

purified crystalline PHB (1, 31, 88; for a recent review on PHA depolymerase assay methods, see reference 35). aPHB, however, is not a physiological substance: all in vivo surface-attached proteins are absent in aPHB. Therefore, it is difficult to predict whether an enzyme with PHA depolymerase activity in vitro using aPHB as substrate also has iPHB depolymerase activity with nPHB granules in vivo. One should consider that the majority of enzymes in living cells are hydrolases and that many of them are esterases. Therefore, hydrolases and/or esterases might exist that have physiological functions other than in PHB metabolism but have the ability to hydrolyze the ester bond of PHB in aPHB in vitro; in vivo, however, they do not hydrolyze nPHB because the enzymes are either located in different cell compartments and/or are not able to bind to the polymer in vivo because the polymer chains are shielded by the phasin layer. The use of cautiously isolated native PHB granules as a substrate for in vitro iPHB depolymerase assay might lead to more meaningful results. For many of the putative iPHB depolymerase genes described in the literature the biochemical activity with nPHB granules has not been experimentally confirmed.

The number of identified putative iPHB depolymerases is markedly increasing with increasing genome sequencing activities, but only for a few iPHB depolymerases do biochemical or in vivo data exist and support the classification as an intracellular nPHB depolymerase, such as the depolymerases of *R. eutropha* (PhaZa1) (23, 88), *P. denitrificans* (15), and *Rhodobacter sphaeroides* (43). A putative intracellular nPHB depolymerase of *R. rubrum* (62) turned out to be a periplasm-located enzyme (24). The physiological function of a PHB depolymerase in the periplasm remained obscure. The maximal activity of this depolymerase was strictly dependent on the pretreatment of nPHB granules with trypsin or an activatorlike proteinaceous compound (25, 26). An iPHB depolymerase unrelated to the previously described ones was recently described for *Bacillus thuringiensis* (112). Again, the iPHB depolymerase activity was dependent on trypsin treatment of nPHB granules. Recently, we identified a new type of iPHB depolymerase ($PhaZ3_{3Rru}$) in *R. rubrum* (accession no 22769.1 [A. Snaidjer and D. Jendrossek, unpublished results]). This depolymerase hydrolyzed nPHB granules in vitro without any activation by trypsin or activatorlike compounds at high rates. Taken together, despite the inflation of putative iPHB depolymerase sequences in the databases, only a small number of them are probably true (physiological) iPHB depolymerases. The most likely candidate for a physiological iPHB depolymerase in *R. eutropha* is PhaZa1 that is the first of all described iPHB depolymerases (88). Although it has no detectable hydrolytic activity when added to isolated nPHB granules and only poor activity with aPHB in vitro (1), PhaZa1 is an nPHB granule-bound protein in vivo (113) and deletion of *phaZa1* resulted in significant less release of 3HB from PHB accumulated cells compared to the wild type. Complementary experiments in which PhaZa1 was expressed in recombinant PHB-accumulating *Escherichia coli* showed increased release of 3HB (114). Surprisingly, the studies of Uchino et al. showed that PhaZa1 can catalyze a thiolysis reaction of nPHB granules in vitro (113). Formation of 3HB-CoA (thiolysis) instead of 3HB (hydrolysis) would save the energy of the ester bond in PHB and could explain why simultaneous synthesis and mobilization of PHB in bacteria did not lead to a futile metabolic cycle (8, 106). Thiolysis experiments using nPHB granules from different bacterial sources as the substrate indicated that the ability to catalyze thiolysis is associated with nPHB granules from some but not all PHB-accumulating bacterial species (unpublished results).

OTHER PHA-ASSOCIATED PROTEINS

It is difficult to assess how many different proteins are localized at the surface of PHA granules in vivo. This is mainly because many proteins can bind artificially to the PHA granule surface during granule isolation. For example, PHB granules isolated from recombinant *E. coli* expressing the PHB biosynthetic *phaCAB* operon of *R. eutropha* have dozens of proteins attached, as revealed by SDS-PAGE analysis of isolated nPHB granules (see Fig. 3 in reference 25). SDS-PAGE analysis of isolated and repeatedly washed *R. eutropha* nPHB granules and subsequent silver staining revealed the presence of a multitude of presumably artificially polymer-bound proteins in addition to PhaP and PhaC (unpublished data). Another example of a protein with strong binding affinity to PHB is lysozyme that is added for the better lysis of cells (51).

PHB synthase was the only protein from which the in vivo subcellular localization at the PHB granule surface was determined about 20 years ago. In the 1990s it became evident that PHA depolymerase and phasin proteins are additional proteins that are constitutively present at the surface of PHB granules (106, 119). Steinbüchel and coworkers were the first to investigate the "proteome" of isolated PHB granules and to notice that multiple phasin proteins and other proteins can be bound to isolated PHB granules in *R. eutropha*. However, the physiological relevance of PHB-bound heat shock protein DnaK, GroEL, and a betaketothiolase (*bkt*) remained unknown. In recombinant *E. coli* expressing the nonnatural BPEC pathway (108), PHA granules strongly bound heat shock protein HspA. Interestingly, HspA could mimic the function of natural phasins in terms of determination of the volume-to-surface ratio of accumulated PHAs (107).

Recently, it was found that nPHB granules isolated from *R. eutropha* were able to catalyze degradation of PHB to acetyl-CoA and also the synthesis of PHB from acetyl-CoA (113). It will be necessary to verify this in vitro result by determination of the subcellular localization of the respective enzymes in vivo. In *P. putida*, one of the two acyl-CoA synthetase (in fusion with GFP) was localized in vivo at PHA granules (87). This finding was in agreement with previous findings that up to seven enzymatic activities were identified in isolated PHA granules in *P. putida* (5, 14, 102): aside from PHA synthase and PHA depolymerase acyl-CoA synthetase, acyl-CoA dehydrogenase, enoyl-CoA hydratase, hydroxyacyl-CoA reductase, and ketoacyl-CoA reductase were reported. Another important finding was the identification of a protein with regulatory function (PhaR) at the PHB granule surface in *R. eutropha* by Western blotting and immunogold labeling in thin sections (see above) (73). All of these results show that more enzymatic functions are localized on PHB granules in vivo than previously assumed (for an overview, see Table 1). Presumably, the degree of subcellular organization in prokaryotes is underestimated.

PHA GRANULE BIOGENESIS

Investigation of PHA metabolism during the last two decades has led to a considerable increase in understanding of the function of individual proteins such as PHA synthases, phasins, PhaR, or PHA depolymerases (e.g., see references 35, 36, 76, 79, 81, 104, and 105 for reviews). However, initiation of PHA synthesis and PHA granule assembly is not understood very well. Analysis by the Sinskey and Stubbe group showed that dimerization of two PHB synthase subunits is required during the initiation process of PHB formation in vitro (38, 105). Recent studies using novel techniques such as atomic force microscopy were able to provide fascinating pictures of a nascent growing PHB chain with terminal attached PHB synthase molecule in vitro (41, 91). Attempts at computer modeling of PHB synthesis were also made (40). However, despite the advantage of in vitro studies for investigation of molecular events occurring during enzymatic catalysis, in vivo investigations on the initiation of PHA granule formation are also necessary for understanding PHA granule assembly. Two models of PHA granule formation have been proposed (104). The micelle model assumes that soluble PHA synthase reacts with substrate molecules (CoA-thioesters of suitable hydroxyalkanoic acids) in the cytoplasm; once the first PHA chains have been synthesized, the polymer molecules aggregate by hydrophobic interactions thus forming nascent small PHA granules. PHA synthase remains attached to the surface of PHA granules and therefore becomes insoluble. Phasin molecules (PhaPs) and other PHA-specific proteins (PhaZs, PhaR) bind to the growing surface of PHA granules. The budding model assumes that PHA synthase is associated with the cytoplasmic membrane and/or that the nascent PHB chain interacts with the cytoplasmic membrane by hydrophobic interactions, resulting in the formation of PHB molecules in or at the cytoplasmic membrane. The granules detach from the membrane at later stages (budding) and PHA-specific surface proteins can be attached to the growing granules. The consequence of the two models is that "early" PHB granules should be membrane bound in the budding model and that PHA granules should be localized in the cytoplasm at all stages of formation in the micelle model. Contradictory results on subcellular localization of "early" PHB granules have been obtained for *R. eutropha*: Tian et al. found "early" PHB granules more or less in the middle of cells near so-called "mediation elements" by transmission electron microscopy analysis (109, 110). Investigation of PHB granules formation by fluorescence microscopy using Nile Red staining suggested that "early" PHB granules are localized in the cell periphery and/or near the cell poles (34). Inspection of other bacteria with larger cell dimensions such as *Caryophanon latum* or *Azotobacter vinelandii* that facilitate differentiation between periphery localization and localization in the cytoplasm confirmed that early PHB granules are often localized in the cell periphery (29, 37). Investigation of recombinant *E. coli* expressing PHA synthase-GFP fusions showed that PHB synthase from *R. eutropha* and PHA synthase from *P. aeruginosa* are both localized at the cell poles (67, 68). Recently, investigation of PHB granule formation in living *R. eutropha* cells by fluorescence microscopy showed that it takes only 10 min after transfer of PHB-free cells to a medium promoting PHB accumulation until first periphery- or cell pole-located granules with attached PhaC-EGFP fusions became visible in living cells: cells of *R. eutropha* transiently accumulate PHB during growth on nutrient broth (NB) medium and fusions of PHB synthase with EGFP were clearly associated with PHB granules as shown by colocalization of green fluorescence and Nile Red fluorescence. However, accumulated PHB was mobilized completely in the stationary growth phase, i.e., after 24 to 36 h of incubation at 30°C. Such PHA-free cells showed green fluorescence distributed in the cells. However, significant fluorescence intensity was also visible along with the cytoplasmic membrane and occasionally in form of very tiny granular spots in the periphery of the cells. Interestingly, the green fluorescence of such stationary cells "migrated" to the cell periphery and aggregated to one to three larger globular structures (foci) near the cell poles or cell periphery within only 5 to 10 min after transfer to a fresh medium permissive for PHB accumulation (Fig. 1). Most cells showed accumulation of GFP fluorescence in the periphery consistent with a membrane stain and in emerging PHB granules. Staining of the cells with Nile Red resulted in weak granular red staining, indicating that these cell pole/periphery-located granules represent early PHB granules. Apparently, the site of PHB granule initiation is specifically controlled in *R. eutropha* and in many other PHB-accumulating bacteria (29, 34, 37, 68). However, proteins that determine the nonrandom localization have not yet been identified. Recently, is was shown that the core region of PHB synthase but not PhaP1 was necessary for the polar localization of PHB synthase (67). One may speculate that the skeletonlike structures that have been observed by atomic force microscopy of isolated PHB granules (7) participate in the determination of the sites of PHB granule

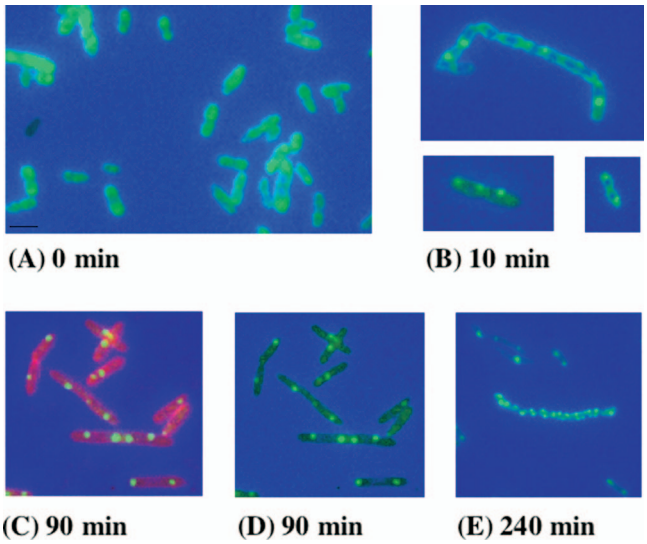

FIG. 1. Formation of PHB granules. A PHB-free culture of *R. eutropha* PHB-4 harboring pBBRMCS2::*phaC-egfp* was transferred to fresh NB medium supplemented with 0.2% sodium gluconate at 0 min. Note the formation of globular PhaC-EGFP foci and the movement of PhaC-EGFP to the cell periphery within only 10 min of incubation at 30°C. The first PHB granules appear after 10 min (Nile Red stain [not shown]). The cells in panel C were stained with the fluorescent dye FM4-64 (FM4-64 is specific for the outer leaflet of cell membranes). Enhanced GFP alone is a soluble protein in *R. eutropha* (not shown).

initiation. Remarkably, atomic force microscopy imaging of PHB granules that had been isolated from *R. eutropha* by digestion with sodium hypochlorite revealed that the granules were arranged in a way that the former cell shapes were still visible (48). This result suggests that hypochlorite-resistant molecules are associated with PHB granules.

CONCLUDING REMARKS

The presence of structural, biosynthetic, catabolic, and even regulatory proteins on PHA granules indicates that PHA granules are complex organized subcellular structures (organelles) for which the designation "carbonosomes"—in analogy to carboxysomes and magnetosomes—is proposed. The designation "carbonosome" should include other prokaryotic storage compounds of carbon and energy aside from PHAs such as granulose (83–85), glycogen (4, 105), triacylglycerols, and wax esters (116, 117). Thus far, several enzymatic activities have been found attached to or associated with the above-mentioned inclusions. With respect to PHAs it will be necessary to identify the target molecules (proteins?) that interact with PHA synthase and/or early PHB granules and that could be responsible for the localization of early PHB granules in the cell periphery. It might be worth investigating the initiation processes of other carbonosomes as well.

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